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(54) Title: METHODS FOR TREATING VASCULAR DISORDERS BY INHIBITING THE ENDOTHELIN STIMULATORY ACTIVITY OF TGFβ

(57) Abstract

The present invention provides methods for preventing or treating a disorder characterized by a deleterious release of endothelin by administering an effective amount of an agent that inhibits the endothelin stimulatory activity of TGFβ. The inhibitory agent can be an anti-TGFβ antibody, decorin or a functional equivalent of decorin. The present methods are particularly useful for treating or preventing cardiovascular diseases. Pharmaceutical compositions containing an inhibitory agent and a pharmaceutically acceptable carrier are also provided.

METHODS FOR TREATING VASCULAR DISORDERS BY INHIBITING THE ENDOTHELIN STIMULATORY ACTIVITY OF TGFß

The present invention was supported in part by Grant No. HL-43154 from the National Institutes of Health. The Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

The present invention generally relates to the treatment of vascular disorders. More specifically, the invention provides methods of inhibiting the endothelin stimulatory activity of TGFß as a means to control the deleterious release of endothelin which can result in various vascular disorders.

Endothelin-1 (ET-1) is a 21 amino acid peptide synthesized by vascular endothelial cells. The peptide has been shown to stimulate vascular, cardiac and bronchial smooth muscle cell growth and contraction. A C-terminal extended form of ET-1, referred to as ET-1^{1-39}, is also synthesized by endothelial cells and is believed to serve as a precursor for ET-1. In contrast to ET-1, ET-1^{1-39} exhibits low biological potency. Two other ET-related peptides have been characterized, ET-2 and ET-3; however, neither of these peptides are produced by vascular endothelial cells.

Endothelin is constitutively released and anatomically distributed throughout the entire vascular system. Only modest increases of plasma concentrations of endothelin have been observed under a variety of physiological and pathophysiological conditions. Based on its anatomic distribution and biological actions, however, endothelin has been hypothesized to play an important role

in the regulation of vascular smooth muscle tone and growth.

Endothelin release following vascular injury may cause the reduction of blood flow and changes of smooth muscle growth that result in the impairment of normal organ function. In addition, the administration of endothelin to animals has been shown to result in an increase of vascular resistance and arterial pressure. Thus, under normal circumstances and pathophysiologic conditions, endothelin is believed to participate in the development of hypertension and abnormalities of vascular wall growth.

In various in vitro studies that have been reported, several factors have been identified that increase endothelin release. Such factors include TGFß, thrombin, vasopressin, angiotensin-II and histamine. Of these factors, TGFß has been identified as the most potent stimulator of endothelin synthesis and release by vascular endothelial cells.

Recent studies have demonstrated that the size of myocardial infarction induced by coronary artery ligation can be significantly attenuated by administration of an endothelin blocking antibody as reported in Watanabe et al., Nature 344:114 (1990). In addition, it has been shown that vascular injuries are often accompanied by a dramatic increase of TGFß in the injured tissues, as reported by Casscells et al., Ann. N.Y. Acad. Sci. 593:148-160 (1990) for myocardial infarction. These studies provide evidence that the post injury-induced reduction of myocardial blood flow may well be mediated by the combined actions of endothelin and $TGF\beta_1$. Under these circumstances, it is possible that the vascular segmental production of $TGF\beta_1$ by platelets, cardiac myocytes or smooth muscle cells may mediate changes of endothelin production resulting in changes of vascular smooth muscle tone or growth.

Although endothelin is believed to participate as a paracrine regulator of vascular function, the physiologic role of the peptide is unknown. To date, there is no known pharmacologic method that inhibits the endothelin stimulatory activity of TGFß. Thus, a need exists to regulate the endothelin stimulatory activity of TGFß in order to prevent or treat vascular disorders characterized by the deleterious effects of endothelin. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The release of endothelin by vascular endothelial cells has been associated with certain vascular disorders, such as myocardial infarction and other cardiovascular diseases. It has been found that TGFß stimulates the production and release of endothelin and that by blocking this stimulatory activity of TGFß, the deleterious effects of endothelin release can be controlled.

The present invention thus provides methods for preventing or treating a disorder characterized by a deleterious release of endothelin by administering an effective amount of an agent that inhibits the endothelin stimulatory activity of TGFß. The inhibitory agent can be an anti-TGFß antibody, decorin or a functional equivalent of decorin. The present methods are particularly useful for treating or preventing cardiovascular diseases.

Pharmaceutical compositions containing an inhibitory agent and a pharmaceutically acceptable carrier are also provided. The compositions are useful in the methods provided by the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the dose-dependent inhibition of decorin on TGFß-induced endothelin release from vascular endothelial cells in culture.

Figure 2 shows the inhibitory effects of a constant dose of decorin (6 µg/ml) on differing doses of TGFß-induced endothelin release.

DETAILED DESCRIPTION OF THE INVENTION

The present invention generally relates to methods of controlling the synthesis and release of endothelin by vascular endothelial cells. The ability of TGFß to regulate the release of endothelin provides a mechanism for controlling such endothelin release. It has now been discovered that blocking the endothelin stimulatory activity of TGFß with antagonists results in inhibiting the release of endothelin. Thus, the inhibition of TGFß's action on endothelial cells can prevent or reduce the production of endothelin release and attenuate the deleterious changes of vascular function that can occur following vascular injury.

The present invention accordingly provides methods for preventing or treating a disorder characterized by the deleterious release of endothelin in an individual. The methods can be accomplished by administering an effective amount of an agent that inhibits the endothelin stimulatory activity of TGFß to the individual.

Various disorders are characterized by the deleterious release of endothelin and can, therefore, be treated or prevented by the methods of the present invention. For example, the present methods can be used to

prevent or treat various cardiovascular disorders such as vasospasm associated with coronary artery disease, vasospasm and smooth muscle proliferation following vascular angioplasty procedures, cerebrovascular spasm associated with stroke and low perfusion states, decreased blood flow to ischemic extremities following surgical procedures such as replant surgery, or deleterious cardiovascular responses to burn injury and edema formation. Such cardiovascular responses include all types of reperfusion, ischemic or occlusion disorders that relate to vasorestriction of smooth muscles. The responses can also be caused by grafts, implants, angioplasty or stroke. The methods can also be used for special cases, such as hypertension, hypertension of pregnancy, raynauds, congestive heart disease or burn injury for example. Disorders associated with or characterized by the deleterious release (i.e., overexpression) of endothelin can be readily determined by methods known in the art or by following the guidance set forth herein.

Agents that inhibit the endothelin stimulatory activity of TGFß include, for example, anti-TGFß antibodies, decorin and functional equivalents of decorin. Other agents having the desired inhibitory activity can also be readily determined by those skilled in the art by testing the agent's ability to block the endothelin stimulatory activity of TGFß as set forth in the examples.

Anti-TGFß antibodies useful in the methods of the present invention can be produced by any method known in the art. The production of monoclonal and polyclonal (i.e. antiserum) antibodies are well known in the art as described, for example, in Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory 1988) which is incorporated herein by reference. Other known methods of producing antibodies or antibody-like compounds are also contemplated. Antibody-like compounds can be, for example,

single chain antibodies or fragments of anti-TGFß antibodies, such as Fab' or F(ab)'$_2$. In general, fragments of such antibodies contain a TGFß binding region that is capable of inhibiting the ability of TGFß to induce endothelin release.

The usefulness of antibodies and antibody-like compounds as the agent has been demonstrated by the administration of a TGFß$_1$ antiserum. The antiserum has been shown to prevent serum-induced endothelin release from bovine vascular endothelial cells in culture as disclosed in Brown et al., Endocrinology 129:2355-2360 (1991), which is incorporated herein by reference. In the studies, one microliter of anti-TGFß$_1$ antiserum completely prevented the increase of endothelin induced by 0.1 ng TGFß$_1$. The results of these studies thus demonstrate that the administration of TGFß$_1$ antiserum to endothelial cells in culture attenuated endothelin release induced by TGFß$_1$. In contrast, the antiserum did not attenuate the release of the C-terminal extended forms of ET-1, specifically ET-1^{1-38} (human) and ET-1^{1-39} (porcine). This finding suggests that the antiserum is specific for inhibiting only the biologically active forms of endothelin, and in particular ET-1.

Alternatively, the agent can be decorin or a functional equivalent of decorin. As used herein, "decorin" refers to a proteoglycan having substantially the structural characteristics described in Krusius & Ruoslahti, Proc. Nat'l Acad. Sci. (U.S.A.) 38:7638 (1986). Human fibroblast decorin has substantially the amino acid sequence described in Krusius & Ruoslahti, supra. "Decorin" also refers to the native composition, the core protein and to modifications of the native proteoglycan that substantially retain the functional characteristics. "Decorin core protein" refers to decorin that no longer is substantially substituted with glycosaminoglycan and is

included within the definition of decorin. Decorin can be rendered glycosaminoglycan-free by enzymatic treatment, mutation or other means, such as by producing decorin in recombinant host cells in which the cells are incapable of attaching glycosaminoglycan chains to a core protein.

Functional equivalents of decorin include modifications of decorin that retain its functional characteristics and molecules that are homologous to decorin, such as the decorin family members biglycan and fibromodulin, for example, that have the similar functional activity of decorin. Such functional equivalents can also include fragments of decorin having similar functional activities of decorin in their ability to inhibit TGFß-induced endothelin release. The fragments can be made by recombinant DNA methods or synthetical using methods known in the art. Modifications of decorin can include, for instance, the addition of one or more side chains or point mutations that do not interfere with the functional activity of the decorin core protein.

Further, both decorin and biglycan are about 80% homologous and contain a leucine-rich repeat of about 24 amino acids in which the arrangement of the leucine residues is conserved. As defined, each repeat generally contains at least two leucine residues and can contain five or more. Proteoglycans and other peptides having structural similarity to decorin, such as fibromodulin, for instance, would be expected to have similar activity and are thus also considered functional equivalents of decorin.

The ability of decorin to block TGFß-induced endothelin release from vascular endothelial cells in culture has been demonstrated. As illustrated in Figures 1 and 2, decorin produced a dose-dependent inhibition of TGFß-induced endothelin release. As shown, 6 µg/ml of decorin resulted in a near complete inhibition of TGFß (0.1

ng)-induced endothelin release.

The present invention further provides pharmaceutical compositions containing an agent that inhibits the endothelin stimulatory activity of TGFß, such as those described above, and a pharmaceutically acceptable carrier. Such pharmaceutically acceptable carriers include, for example, hyaluronic acid and aqueous solutions such as bicarbonate buffers, phosphate buffers, Ringer's solution and physiological saline supplemented with 5% dextrose or human serum albumin, if desired. Other pharmaceutical carriers known to those skilled in the art or later developed are also contemplated. The pharmaceutical compositions can also include other reagents that are useful for the prevention or treatment of the various disorders. Those skilled in the art can readily identify such reagents. In treating specific vascular disorders, various reagents, such as angiotensic converting enzyme inhibitors, heparin, aspirin, ß-adrenic receptor antagonists or nitrates, can be included in the pharmaceutical compositions.

The pharmaceutical compositions can be used in the methods of the present invention. Such compositions or other formulations containing the TGFß antagonists (agents) can be administered to a patient in a number of ways, including, for example, intravenously or orally if using stabilized analog.

The following examples are intended to illustrate but not limit the invention.

EXAMPLE I

Preparation of Endothelial Cells

Bovine aortic endothelial cells were prepared, cloned, and maintained by methods previously described in

Gospodaraowicz et al., J. Cell Physiol. 122:323-332 (1985), and in Gospodaraowicz et al., Endocrinology 118:82-90 (1986), both incorporated herein by reference. Cells were grown to confluence in 24 well dishes and 1 ml HEPES (25 mM) buffered Delbeco's Modified Eagles Media (DMEM) supplemented with 10% calf serum. Prior to addition of test substances, cells were washed twice with DMEM and the culture media was replaced with DMEM-containing 0.1% bovine serum albumin.

EXAMPLE II

Treatment of TGFß, with Decorin

Decorin in the concentrations indicated in Figures 1 and 2 was incubated with TGFß for one hour prior to its addition to the cells. All experiments were performed in serum-free media. The culture media were collected 18 hours later and assayed for endothelin immunoactivity as described in Example III. Each experiment was performed at least twice, with 4 to 6 wells allocated to each treatment.

The results of these experiments are reported in Figures 1 and 2. Data are presented as mean ± SEM. Group differences were determined using one-way analysis of variance followed by the tests of Dunnett and Duncan. As shown in the figures, decorin produced a dose-dependent inhibition of TGFß-induced endothelin release, with 6 µg/ml of decorin resulting in a near complete inhibition of TGFß (0.1 ng)-induced endothelin release.

EXAMPLE III

Endothelin Radioimmunoassay

ET concentrations in 50 µl samples of tissue culture fluid were measured using a specific radioimmunoassay described in Hexum et al., Biochem.

Biophys. Res. Commun. 167:294-300 (1990), incorporated herein by reference. I^{125}-endothelin was prepared using the conventional chloramine-T method and subsequently purified on Bond Elute C_{18} columns (Analytichem International, Harbor City, CA) as described in Example IV. Radioimmunoassays were performed using a buffer containing 0.05 M sodium phosphate, 0.15 M sodium chloride, 0.25 M sodium EDTA, 0.25% BSA, and 0.1% sodium azide at pH 7.4. Separation of free from bound tracer was performed using goat anti-rabbit serum (Wylie Vale, Salk Institute). This ET radioimmunoassay equally recognizes ET-1, ET-2, and ET-3 but does not cross-react with $ET\text{-}1^{1\text{-}39}$. The intra- and inter-assay variabilities were 8% and 10%, respectively. The amino acid sequences of ET-1, ET-2, ET-3, $ET\text{-}1^{1\text{-}38}$ and $ET\text{-}1^{1\text{-}39}$ are provided in Table 1.

Table 1

Endothelin	Amino Acid Sequence	SEQ. ID NO.
ET-1	CYS-SER-CYS-SER-SER-LEU-MET-ASP-LYS-GLU-CYS-VAL-TYR-PHE-CYS-HIS-LEU-ASP-ILE-ILE-TRP	1
ET-2	CYS-SER-CYS-SER-SER-TRP-LEU-ASP-LYS-GLU-CYS-VAL-TYR-PHE-CYS-HIS-LEU-ASP-ILE-ILE-TRP	2
ET-3	CYS-THR-CYS-PHE-THR-TYR-LYS-ASP-LYS-GLU-CYS-VAL-TYR-TYR-CYS-HIS-LEU-ASP-ILE-ILE-TRP-	3
$ET\text{-}1^{1\text{-}38}$ (human)	CYS-SER-CYS-SER-SER-LEU-MET-ASP-LYS-GLU-CYS-VAL-TYR-PHE-CYS-HIS-LEU-ASP-ILE-ILE-TRP-VAL-ASN-THR-PRO-GLU-HIS-VAL-VAL-PRO-TYR-GLY-LEU-GLY-SER-PRO-ARG-SER	4
$ET\text{-}1^{1\text{-}39}$ (porcine)	CYS-SER-CYS-SER-SER-LEU-MET-ASP-LYS-GLU-CYS-VAL-TYR-PHE-CYS-HIS-LEU-ASP-ILE-ILE-TRP-VAL-ASN-THR-PRO-GLU-HIS-VAL-VAL-PRO-TYR-GLY-LEU-GLY-SER-PRO-SER-ARG-SER	5

EXAMPLE IV

Chromatography

Serum containing endothelin was purified on Bond Elut C_{18} columns containing 500 mg sorbent. Columns were wetted using 50% 2-propanol/50% TEAF (11.5 ml 88% formic acid/liter glass-distilled water, pH adjusted to 3.0 with triethylamine). Samples were mixed with an equal amount of TEAF and applied to the column. No greater than 1 ml serum was applied to each column. columns were washed with 3 ml TEAF, and endothelin-releasing activity was eluted with 1 ml 50% 2-propanol/50% TEAF to the column. After collection, samples were lyophilized and reconstituted in 100 µl DMEM.

Although the invention has been described with reference to the presently-preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: LA JOLLA CANCER RESEARCH FOUNDATION

(ii) TITLE OF INVENTION: METHODS FOR TREATING VASCULAR DISORDERS BY INHIBITING THE ENDOTHELIN STIMULATORY ACTIVITY OF TGFB

(iii) NUMBER OF SEQUENCES: 5

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Ser Cys Ser Ser Leu Met Asp Lys Glu Cys Val Tyr Phe Cys His
1 5 10 15

Leu Asp Ile Ile Trp
20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Cys Ser Cys Ser Ser Trp Leu Asp Lys Glu Cys Val Tyr Phe Cys His
1 5 10 15

Leu Asp Ile Ile Trp
20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Cys Thr Cys Phe Thr Tyr Lys Asp Lys Glu Cys Val Tyr Tyr Cys His
1 5 10 15

Leu Asp Ile Ile Trp
20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Cys Ser Cys Ser Ser Leu Met Asp Lys Glu Cys Val Tyr Phe Cys His
1 5 10 15

Leu Asp Ile Ile Trp Val Asn Thr Pro Glu His Val Val Pro Tyr Gly
20 25 30

Leu Gly Ser Pro Arg Ser
35

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Cys Ser Cys Ser Ser Leu Met Asp Lys Glu Cys Val Tyr Phe Cys His
1 5 10 15

Leu Asp Ile Ile Trp Val Asn Thr Pro Glu His Val Val Pro Tyr Gly
20 25 30

Leu Gly Ser Pro Ser Arg Ser
35

We claim:

1. A method for preventing or treating a disorder characterized by a deleterious release of endothelin in an individual, comprising administering to said individual an effective amount of an agent that inhibits the endothelin stimulatory activity of TGFß.

2. The method of claim 1, wherein said disorder is a cardiovascular disease.

3. The method of claim 1, wherein said disorder is a vascular disease, injury or reperfusion.

4. The method of claim 1, wherein said agent is decorin.

5. The method of claim 1, wherein said agent is a functional equivalent of decorin.

6. The method of claim 5, wherein said functional equivalent is biglycan.

7. A pharmaceutical composition comprising an agent that inhibits the endothelin stimulatory activity of TGFß and a pharmaceutically acceptable carrier.

8. The pharmaceutical composition of claim 7, wherein said agent is decorin.

9. The pharmaceutical composition of claim 7, wherein said agent is a functional equivalent of decorin.

10. The pharmaceutical composition of claim 9, wherein said functional equivalent is biglycan.

FIGURE 1

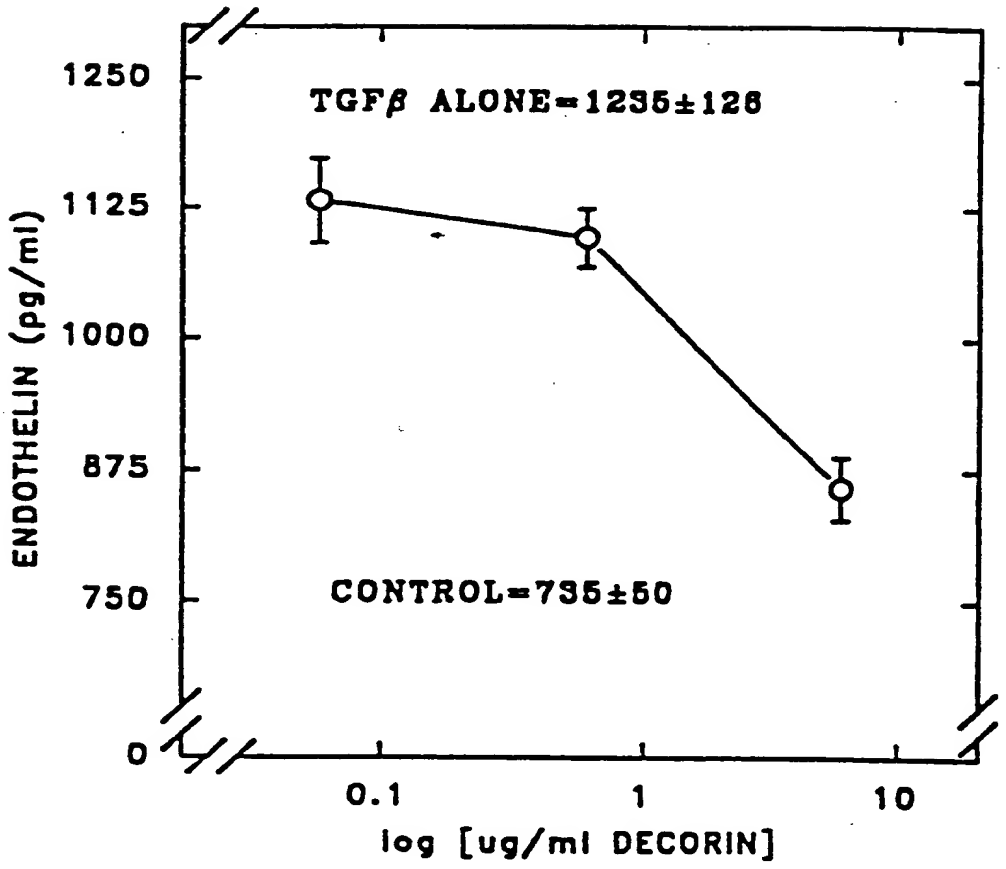
1250
1125
1000
875
750
0
ENDOTHELIN (pg/ml)
TGFβ ALONE=1235±126
CONTROL=735±50
0.1
1
10
log [ug/ml DECORIN]

FIGURE 2

EFFECTS OF DECORIN ON TGFB-INDUCED ENDOTHELIN RELEASE